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Induction of Inflammatory Cytokines and Interferon Responses by Double-stranded and Single-stranded siRNAs is Sequence-dependent and Requires Endosomal Localization

Mouldy Sioud

The Norwegian Radium Hospital, Department of Immunology, Molecular Medicine Group, Montebello N-0310 Oslo, Norway

The potential induction of inflammatory cytokines and interferon responses by small-interfering RNAs (siRNAs) represents a major obstacle for their use as inhibitors of gene expression. Therapeutic applications of siRNAs will require a better understanding of the mechanisms that trigger such unwanted effects, especially in freshly isolated human cells. Surprisingly, the induction of tumor necrosis factor (TNF-α) and interleukin-6 (IL-6) in adherent peripheral blood mononuclear cells (PBMC) was not restricted to double-stranded siRNAs, because induction was also obtained with single-stranded siRNAs (sense or antisense strands). The immunostimulatory effects were sequence-dependent, since only certain sequences are prone to induce inflammatory responses while others are not. The induction of TNF-α, IL-6 and interferon α (IFN-α) was chloroquine-sensitive and dependent more likely on endosomal Toll-like receptor signaling in particular TLR8. Indeed, no significant immuno-stimulatory effects were detected when either double or single-stranded siRNAs were delivered directly to cytoplasm *via* electroporation. Both RNA types activated a NF-κB promoter-driven luciferase gene in transiently transfected human adherent PBMC. Moreover, culture of immature dendritic cells with either double or single-stranded siRNAs stimulated interleukin-12 production and induced the expression of CD83, an activation marker. Interestingly, several double-stranded siRNAs did not induce TNF-α, IL-6 and IFN-α production, however, their single-stranded sense or antisense did. Taken together, the present data indicate for the first time that the induction of inflammatory cytokines and IFN-α responses by either double-stranded or single-stranded siRNAs in adherent PBMC is sequence-dependent and requires endosomal intra-cellular signaling. The finding that endosomal localization of self-RNAs (sense strands) can trigger Toll-like receptor signaling in adherent human PBMC is intriguing because it indicates that endosomal self-RNAs can display a molecular pattern capable for activating innate immunity.

Keywords: RNA interference; siRNAs; innate immunity; Toll-like receptors; inflammatory cytokines

Introduction

RNA interference (RNAi) is an evolutionarily conserved post-transcriptional gene-silencing process, resulting in specific mRNA degradation in several organisms.[1,2] In this process, long double-stranded (ds) RNAs were processed by the RNase III-like enzyme Dicer to 21–25 nucleotides small-interfering RNAs (siRNAs), which are then incorporated into a protein complex, the RNA-induced

Abbreviations used: DC, dendritic cells; iDC, immature dendritic cells; siRNA, small-interfering RNA; RNAi, RNA interference; PKR, protein kinase R; TLR, Toll-like receptor; IL, interleukin; TNF-α, tumor necrosis factor α; NF-κB, nuclear factor kappa B; INF, interferon; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethyl-ammoniummethyl-sulfate; PBMC, peripheral blood mononuclear cells.

E-mail address of the author: mosioud@ulrik.uio.no

silencing complex (RISC). Thereafter, the RISC is remodulated into its active form, which contains the proteins necessary for cleaving the target mRNA at the site, where the guide antisense siRNA strand binds.[2] RNAi and the related phenomenon of quelling and post-transcriptional gene silencing (PTGS) have been shown to exist in fungus (Neurospora), plants (Arabidopsis), invertebrates (*Drosophila* and *C. elegans*) and embryonic mouse and human cells.[2,3] Notably, the cellular role of RNAi is to maintain the integrity of the genome, to defend cells against viral infection, and to regulate the expression of cellular genes.

In general RNAi was difficult to detect in somatic mammalian cells, since dsRNA structures greater than 30 bp stimulate the IFN pathway, which represents a host response to viral infection where several genes are activated.[4] Much of the interferon responses are caused by the activation of the dsRNA-dependent protein kinase R (PKR). The antiviral effect of PKR is in part mediated through the phosphorylation of the alpha subunit of the eukaryotic translation initiation factor eIF2α, which results in the sequestration of the recycling factor eIF-2β in an inactive complex together with eIF2α-GDP.[5] The net effect is global inhibition of protein synthesis. However, it has recently been shown that synthetic 21 nt siRNAs, the effector in RNAi, transfected into human somatic cells can effectively bypass activation of the interferon pathway.[6] Since then siRNAs have become a powerful tool for genetic analysis and might serve as a potent therapeutic tool for gene silencing.[7,8] In addition to mRNA cleavage, siRNAs can induce chromatin modification in different organisms. Interestingly, a recent report specified a function for siRNAs in DNA methylation in mammalian cells.[9] Contrary to what was thought, recent experiments indicate that exogenously delivered siRNAs can activate the interferon pathway.[10–15] However, there are conflicting reports regarding the extent of off-target effects and interferon induction by siRNA in mammalian cells. For example, Rossi and colleagues noted that none of the three tested chemically made siRNAs induced interferon responses in human cells.[16] These differences could be due to several factors, including differences in the cell types, reagent preparations and siRNA sequences. Notably, in all studies only a few siRNAs were tested using the same experimental conditions. To further address the question of what determines siRNA immuno stimulatory function, a comprehensive analysis of 32 siRNAs targeting different genes was performed. The results indicate that the stimulating capacities of either double-stranded, single-stranded sense or antisense siRNAs are sequence-dependent and require endosomal compartments for intracellular signaling.

Table 1. Sequences of the siRNAs used in this study

SiRNAs	Sequence (5′–3′) (sense strand)	Target gene
1	GGCCUUCCUACCUUCAGACTT	Mouse TNF-α
2	GAUCAUCUUCUCAAAAUUCTT	Mouse TNF-α
3	GUUCACCUGAGCCUAAUAGTT	Human HIF-1
4	CUGAUGACCAGCAACUUGATT	Human HIF-1
5	GACAACCAACUAGUGGUGCTT	Mouse TNF-α
6	GAGGCUGAGACAUAGGCACTT	Mouse TNF-α
7	GAACUGAUGACAGGGAGGCTT	Scrambled siRNA
8	GAAGAAGUCGUGCUGCCUUTT	Scrambled siRNA
9	GGUGACAAGAACAUCUCCATT	Human neuropilin-1
10	GACCUCAUGUACCACAUUCTT	Human PKC-α
11	GCCAUUGCACUGUGAAUACTT	Mouse Csf-1
12	GUGAUCAUUCAGAGCCAGCTT	Mouse Csf-1 receptor
13	GGCAUCUGGCUUAAGGUGATT	Mouse Csf-1 receptor
14	GACCCUCGAGUCAACAGAGTT	Mouse Csf-1
15	GCAUGCCUUGGAAUUCCUUTT	Scrambled siRNA
16	GGCCGAUUGAUCUCAGCGCTT	Human TNF-α
17	GGCCAAAUUUACAUUCUCCTT	Rat NG-2
18	CCAACUAUGACCAGGAUAATT	Human MMP-9
19	GUAGAUCAAUACCCUACACTT	Rat NG-2
20	GGAGCGCACCAUCUUCUUCTT	GFP
21	GACUUGAGCGAGCGCUUUUTT	Human C20orf55
22	GAGAUGAUACCACCUGAAATT	Human CSPP
23	GCAGAUCUGCGUCGGCCAGTT	Human Frizzled-2
24	GGUUCCAUCGAAUCCUGCATT	Human Wnt-1
25	GAGGCAAUCACCAAUAGCATT	Mouse Basigin
26	GAAGAUUUGCGCAGUGGACTT	Human CSPP
27	GUCCGGGCAGGUCUACUUUTT	Mouse TNF-α
28	GGCAUGGAUCUCAAAGACATT	Mouse TNF-α
29	CCAACGGCAUGGAUCUCAATT	Mouse TNF-α
30	UGCCCUUCUACAACCAGGATT	Human AKT-1
31	GCUGGAGUACAACUACAACTT	GFP
32	GCUGGAGAUCCUGAAGAACTT	Human cDNA FLJ34902

Results and Discussion

The non-specific effects of siRNAs are sequence-dependent

siRNAs were initially thought to be small enough to avoid double-stranded RNA responses.[6] However, recent studies indicated that they could induce interferons and cytokine responses.[10–15] Although the reported data provide a note of caution, the activation of the non-specific pathways by siRNAs is not well understood. We have previously examined the response of BALB/c mice to systemic delivery of siRNAs and found induction of TNF-α and IL-6 by larger RNAs and LPS, but not with a chemically synthesized siRNA,[15] whilst the same siRNA preparation induced inflammatory cytokine responses in adherent PBMC *in vitro*.[15] However, further analysis has indicated that some siRNA sequences can induce cytokines and interferon responses in mice (unpublished results). To assess

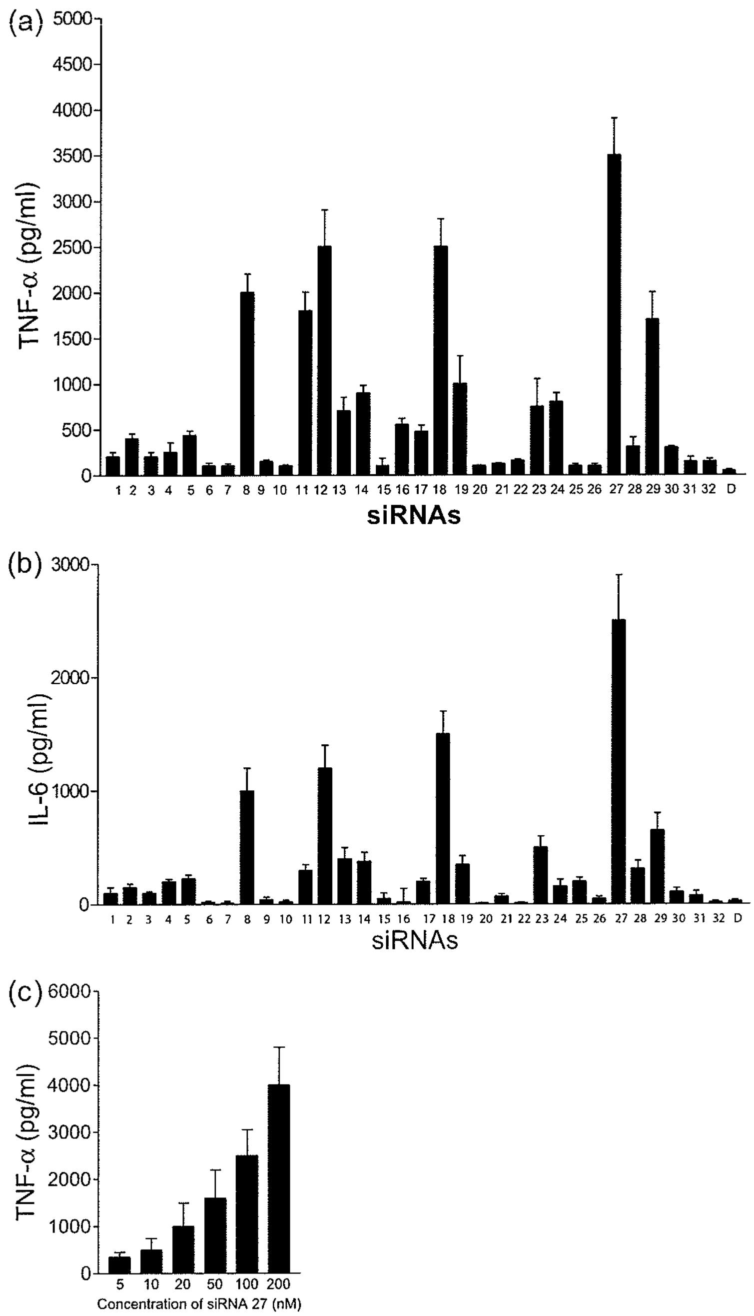

Figure 1. Effects of siRNAs upon TNF-α and IL-6 production in adherent PBMC. Cells were transfected with the indicated siRNA molecules (100 nM) for 18 hours and then TNF-α (a) and IL-6 (b) were measured in the supernatants by ELISA. Results are shown as means of five independent experiments ±SD. The sequences of the used siRNAs are shown in Table 1. (c) Immunostimulatory siRNA induce TNF-α production in a dose-dependent manner. Adherent PBMC were transfected with various concentrations (5–200 nM) of siRNA 27 for 18 hours. Subsequently, TNF-α was measured in the supernatants by ELISA. All results represent the mean of four or more independent experiments. D, cells were incubated with only DOTAP and transfection buffer.

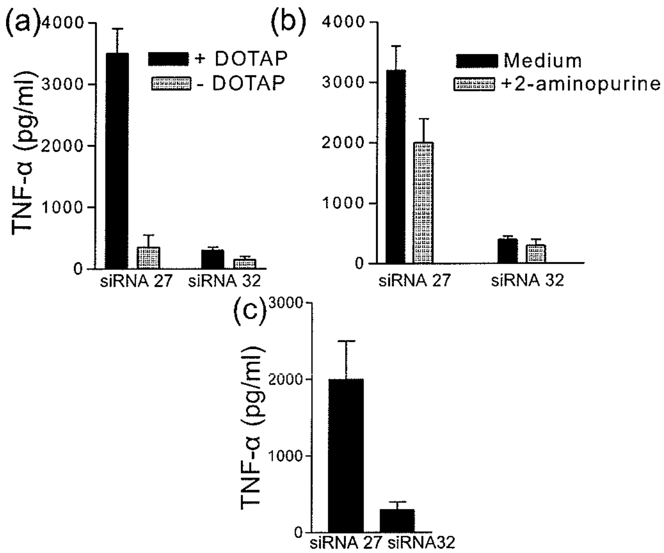

Figure 2. Characterization of siRNA effects. (a) siRNA induced TNF-α production required liposomal delivery of siRNAs. Adherent PBMC were incubated for 18 hours with either siRNA liposome complexes or naked siRNA 27 (100 nM) in X-VIVO 15 medium, a nuclease-free medium. Subsequently, TNF-α was measured in the supernatants by ELISA. Results are shown as means of four independent experiments ±SD. (b) Inhibition of PKR. Adherent PBMC were pretreated, or not, with 2-aminopurine (10 mM) for one hour prior to incubation with either siRNA 27 or 32 (100 nM) for 18 hours. Subsequently, TNF-α was measured in the supernatants by ELISA. Results are shown as means of four independent experiments ±SD. (c) siRNA induced TNF-α production in blood monocytes. Purified CD14-positive cells were incubated with either siRNA 27 or 32 (100 nM) for 18 hours, and then TNF-α was measured in the supernatants using ELISA. All results represent the mean of four or more independent experiments.

whether the siRNA non-specific effects are sequence-dependent and to uncover the molecular mechanisms by which siRNAs, activate innate immuniy genes, the induction of TNF-α and IL-6 secretion by 32 different siRNAs was examined in adherent PBMC. Freshly isolated cells were treated with the various siRNA sequences (see Table 1) for 18 hours. Specific ELISA on culture supernatants revealed that around 50% of the tested siRNAs induced the production of TNF-α (Figure 1(a)). Adherent PBMC produced also IL-6 upon stimulation with siRNAs complexed to DOTAP (Figure 1(b)). The profile of IL-6 production is comparable to that of TNF-α. Notably, siRNAs varied dramatically in their ability to induce TNF-α and IL-6 production, suggesting that their activities are sequence-dependent. Only six of the siRNAs examined exhibited a strong immunostimulatory effect, with siRNA 27 being the most effective under our experimental conditions.

To determine the immunostimulatory capacity of siRNAs, cells were transfected with siRNA 27 at a range of concentrations (5–200 nM). After 18 hours in culture, secreted TNF-α was assessed by ELISA (Figure 1(c)). As shown, siRNA 27 stimulated TNF-α production even at low concentrations. Taken together these data indicate that siRNAs can activate the expression of inflammatory cytokines, but their affinity and specificity for a potential cellular target are more likely to be influenced by the siRNA base composition. Sequence alignment of the siRNA sequences revealed no significant homology between the immunostimulatory siRNAs, suggesting the involvement of RNA tertiary structures and/or specific dinucleotides. Although several immunostimulatory siRNAs contained the dinucleotide GU, which should be avoided during siRNA design, the overall analysis indicates that they are required for the activation of innate immunity genes.

Intracellular siRNA delivery is required for cytokine induction

Having found that the non-specific effects of siRNAs are sequence-dependent, I have next investigated whether intracellular siRNA delivery was required. Therefore siRNAs were added directly to the culture medium without complexing them to DOTAP. siRNA 27 activated adherent PBMC to secrete TNF-α when complexed to DOTAP. In contrast, free siRNA did not lead to significant cytokine production (Figure 2(a)). A nuclease-resistant siRNA 27 gave similar results as its unmodified version (data not shown). These results argue that siRNAs target mainly intracellular compounds. In this respect, a role for PKR in the activation of interferons by siRNAs was recently suggested.[12]

To explore the involvement of the dsRNA recognition protein PKR in this process, the specific inhibitor of PKR, 2-aminopurine, was used. Cells were pretreated with 2-aminopurine (10 mM), a normally used concentration, and subsequently treated with siRNA liposome complexes. Pretreatment with 2-aminopurine reduced, but did not abolish cytokine production by siRNAs (Figure 2(b)). These results would indicate the involvement of other cellular targets in siRNA intracellular signaling.

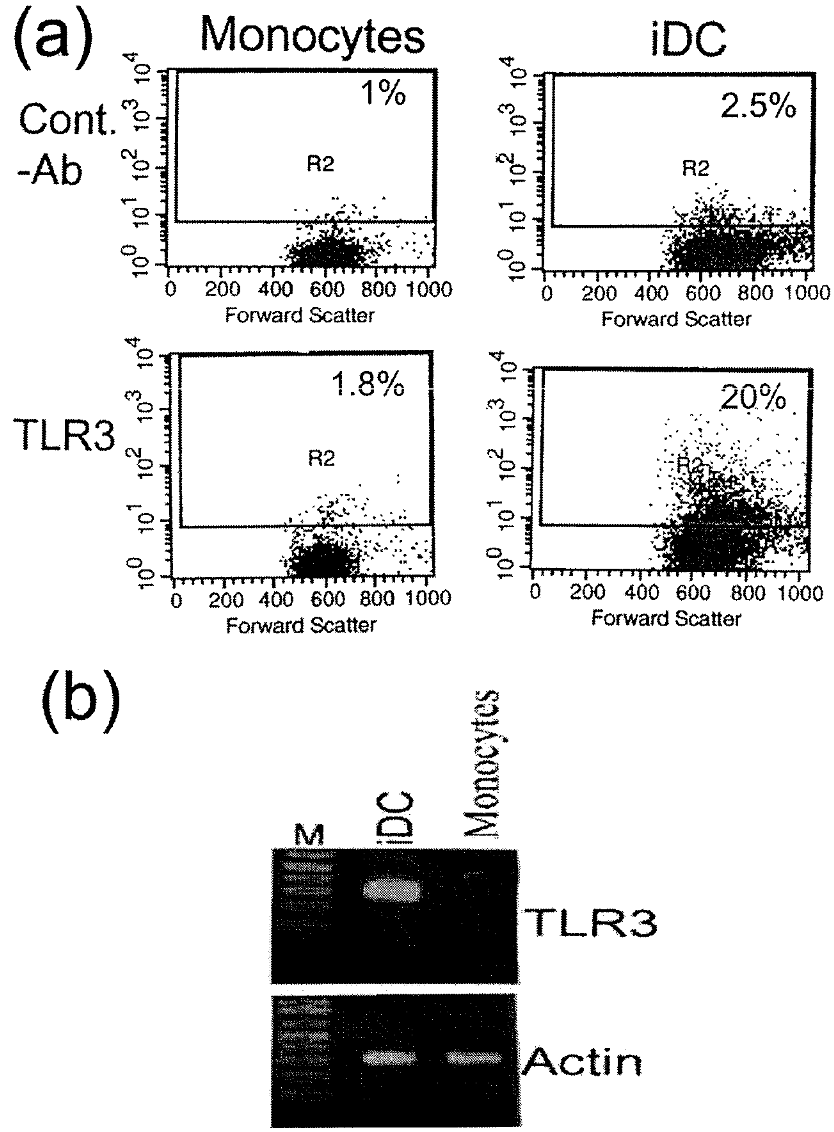

Figure 3. Expression of TLR3 by human monocytes and iDC. (a) Flow cytometry analysis. Freshly isolated monocytes and iDC, monocytes cultured for six days in the presence of GM-CSF and IL-4, were stained either with FITC-labeled anti-TLR3 monoclonal antibody (eBioscience) or control mouse IgG1. After washing, cells were analyzed by flow cytometry. The mean fluorescence of stained cells is shown in the upper-right corner of the individual dot plots. (b) RT-PCR for TLR3 and actin mRNA was performed with monocytes and iDC as described in Experimental Procedures. TLR3 was not detected in human monocytes. PCR analysis revealed the expression of TLR1, 2, 4, 5, 6 and 8 by human monocytes.

siRNAs activate TNF-α production in purified human monocytes

Monocytes are essential effector cells in chronic inflammatory disorders and infectious diseases. To perform their function, monocytes need to be activated, either *via* inflammatory cytokines produced by the adaptive immune system or *via* direct stimulation with microbial products. Although a high proportion of the adherent PBMC are monocytes, the capacity of siRNAs to activate TNF-α production in purified blood monocytes was investigated. In these experiments, CD14-positive cell populations were prepared from PBMC by positive selection with immunomagnetic beads coated with anti-CD14 monoclonal antibodies (Dynal, Oslo, Norway), and the cells were subsequently incubated with siRNAs (Figure 2(c)). There was a significant induction of TNF-α production with the immunostimulatory siRNA 27, whereas no significant activity was seen with the control siRNA 32.

TLR3 is not required for responsiveness to either double-stranded or single-stranded siRNAs in adherent PBMC

Notably, the host sensors that initially detect viral and bacterial antigens and trigger cytokine production have been investigated by several groups, some of which have indicated the involvement of Toll-like receptors (TLRs). These receptors, which are mammalian homologous of the *Drosophila* Toll, recognize specific structural motifs expressed by microbes.[17–19] So far, ten TLRs have been described in humans, and ligands have been defined for nine of them. TLR1, 2 and 6 are triggered by peptidoglycan and other microbial products, TLR3 by dsRNA, TLR4 by LPS, TLR5 by flagellin, TLR7 and 8 by imidazoquinolines, and TRL9 by unmethylated CpG DNA motifs. Although TLR3 is a receptor for dsRNA and cellular mRNA,[20,21] it does not contain dsRNA binding motifs. While this work was underway, a recent study has indicated the involvement of TLR3 in siRNA signaling and suggested the involvement of dsRNA-binding protein that has yet to be identified.[14] In contrast to macrophages and dendritic cells, however, human monocytes do not express TLR3 as assessed by flow cytometry and RT-PCR (Figure 3(a) and (b)). These results agree with those of a recent study reporting on the expression of TLR3 by human monocytes.[22] In addition, treatment of either adherent PBMC or purified monocytes with anti-TLR3 monoclonal antibody, known to block TLR3 signaling,[23] did not inhibit the production of TNF-α by immunostimulatory siRNAs (data not shown). As detected by PCR, monocytes express TLR1, 2, 4, 5, 6 and 8. Because monocytes responded to siRNA by producing TNF-α (Figure 2 (c)), the immunostimulatory capacity of siRNAs is more likely mediated by endosomal Toll-like receptor 8 (see below).

Sense and antisense siRNA strands are highly stimulatory when compared to their double-stranded siRNA counterparts

The data described above indicate that a subgroup of double-stranded siRNAs can induce the production of inflammatory cytokines. During this study, however, I have been surprised by the finding that both single-stranded sense and antisense siRNAs can trigger the production of pro-inflammatory cytokines (e.g. TNF-α) and interferons (IFN-α) in adherent PBMC (Figure 4 as a representative example). This is in contrast to what have been reported using various cancer cell lines.[12,13] As for the double-stranded siRNAs, the induction of TNF-α by single-stranded siRNAs was sequence-dependent. In the case of siRNA 27, the sense strand exhibited the strongest effects, whereas in the case of siRNA 32 the antisense strand exerted the strongest effect on TNF-α production (Figure 4(a)). Notably, the single-stranded siRNAs were highly immunostimulatory compared to their double-stranded siRNA counterparts (e.g. siRNA

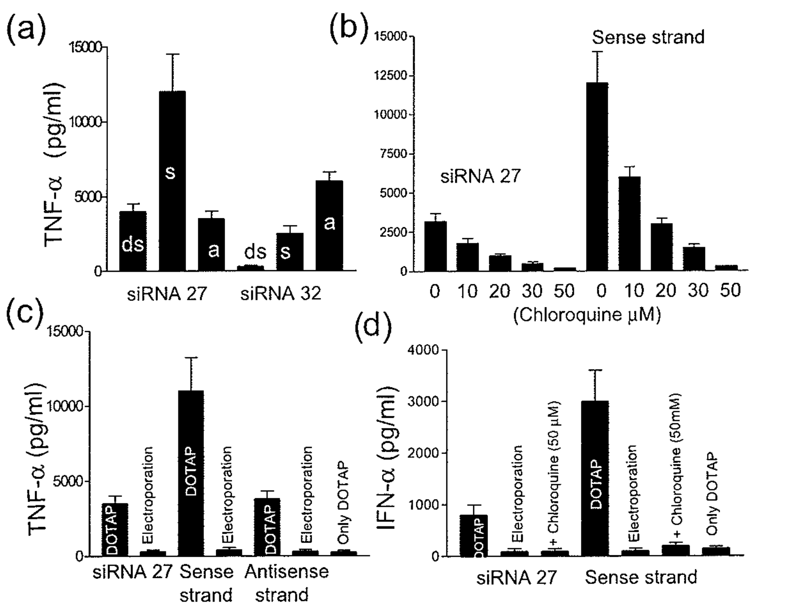

Figure 4. Effects of double-stranded and single-stranded siRNAs upon TNF-α production in adherent PBMC. (a) Cells were transfected with either double-stranded siRNAs (d), sense siRNA strands (s) or antisense siRNA strands (a) for 18 hours. Subsequently, TNF-α was measured in the supernatants by ELISA. All test RNA molecules were used at 100 nM and complexed with DOTAP at 10 μg/ml. (b) Inhibition of cytokine production by chloroquine in dose-dependent manner. Cells were pre-incubated with chloroquine for two hours prior to transfection with siRNAs (100 nM) for 18 hours. Subsequently, TNF-α was measured in the supernatants by ELISA. (c) The test RNA molecules (100 nM) were delivered to the cells either *via* DOTAP or electroporation as indicated on the Figure. After 18 hours transfection time, TNF-α was measured in the supernatants by ELISA. (d) Induction of IFN-α by double-stranded and single-stranded siRNAs. The test molecules (100 nM) were delivered to adherent PBMC *via* either DOTAP or electroporation. To test the involvement of endosomes in siRNA induction of interferons, cells were also pretreated for two hours with chloroquine prior to transfection with DOTAP. After transfection (20 hours), IFN-α was measured in the supernatants by ELISA. All results represent the mean of three or more independent experiments.

32). As shown, DOTAP alone did not induce either TNF-α or IFN-α production, indicating that the effects are related to the RNA sequences. Direct addition of either naked double-stranded siRNAs or single-stranded siRNAs did not affect cytokine production (data not shown). Moreover, several double-stranded siRNAs did not induce response, whereas their corresponding single-stranded sense or antisense strands did (Figure 4(a), as an illustration). It is worth noting that the same single-stranded siRNA preparations were used to prepare the annealed siRNAs. Taken together, these observations would indicate that the reported siRNA effects in adherent PBMC are related to the siRNA sequences and argue against any possible contamination with endotoxins.

Cytokine induction by double-stranded siRNAs, sense or antisense siRNA strands requires endosomal acidification

Cationic liposome-delivered siRNAs are expected to enter the cell *via* endocytosis. Because several Toll-like receptors, in particular TLR7, TLR8, TLR9, are localized in the endosomes, I hypothesized that endocytically introduced RNA could trigger the activation of these receptors, leading to MAPK kinases and NF-κB activation. To test the involvement of endosomes in siRNA signaling, cells were treated with an inhibitor of lysosomal acidification, chloroquine for two hours prior to transfection with either double-stranded or single-stranded siRNAs. TNF-α secretion by adherent PBMC were inhibited in a dose-dependent manner by chloroquine (Figure 4(b)), indicating that the acidification of the endosomes is important for cellular response to either double-stranded or single-stranded siRNAs. A similar inhibition was obtained with Bafilomycin, an additional inhibitor of endosomal acidification (data not shown).

Cationic liposomes such as DOTAP both protect the siRNA and mediate internalization *via* endocytosis. After entry, the siRNA must escape the endolysosomes and enter the RNAi pathway. The requirement for acidification of endosomes in adherent PBMC was further examined *via* electroporation of siRNAs into adherent PBMC. In contrast to DOTAP, the electroporation method opens up pores in the cell, so siRNA molecules can enter the cell directly into the cytoplasm. As shown in Figure 4(c) and (d), no TNF-α and IFN-α induction was obtained when either double-stranded or single-stranded siRNAs were delivered *via* electroporation. Therefore, endosome compartments are required for both double-stranded and single-stranded siRNA intracellular signaling and subsequent cytokine and interferon production by adherent PBMC. Notably, pretreatment of PBMC with chloroquine prior to transfection with siRNA abrogated IFN-α production (Figure 4(d)), indicating for the first time the involvement of endosomes in siRNA induction of interferons. The inhibition of interferon and cytokine responses in adherent PBMC was achieved even at low concentration of chloroquine (1–5 μM).

Endosomal siRNA signaling supports the involvement of the Toll-like receptors 7 and 8

Toll-like receptors (TLRs) represent a class of pattern recognition receptors (PPRs) that detect microbes or their components, which are referred to as pathogen associated molecular patterns (PAMPs).[19] TLRs are predominantly expressed on

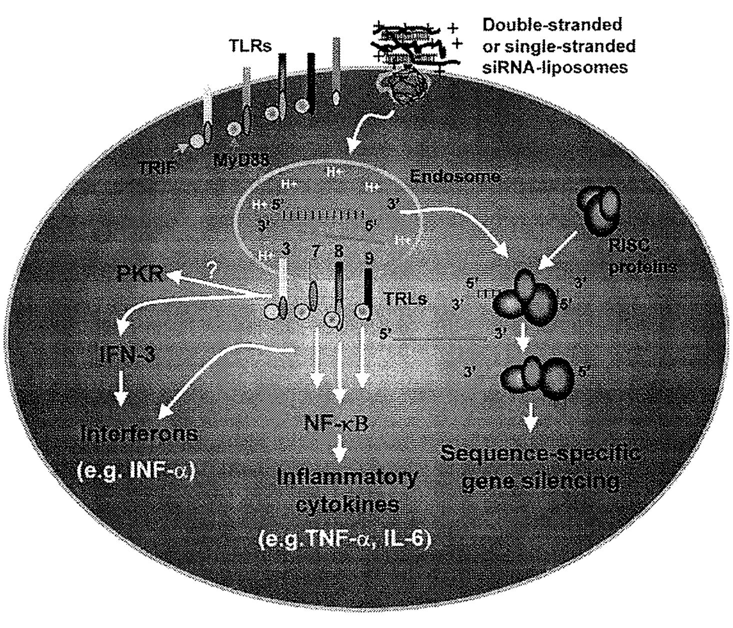

Figure 5. A schematic diagram illustrating the intracellular siRNA signaling. Cationic lipid siRNA complexes bind to the plasma membrane electrostatically and are internalized *via* endocytosis. Within the endosomes RNA molecules can bind to endosomal Toll-like receptors, in particular TLR8 and 7, which can activate the transcription factor NF-κB, leading to inflammatory cytokine production. TLR7, 8 and 9 use MyD88 as primary adaptor to activate interferon production *via* undefined signaling intermediates. TLR3 uses the adaptor protein TRIF to activate interferon regulatory factor-3 (IRF-3). The activation of Toll receptors may activate PKR directly.[19,11] After endosomal escape and subsequent incorporation into RISC complex, siRNAs can trigger sequence-specific gene silencing.

the cell surface; however, a subset (TLR7, TLR8, TLR9, and in some cases, TLR3) is retained in intracellular compartments. Notably, TLR9, TLR7 and TLR8 require endosomal acidification for signaling.[19] Because the induction of inflammatory cytokines by siRNAs is chloroquine-sensitive, the data would support the involvement of these receptors in siRNA intracellular signaling. The recent identification of single-stranded viral RNAs as a ligand for TLR7 and 8[24,25] would support our initial observation[15] and the present elaborated results suggesting the involvement of endosomal TLRs in single-stranded siRNA signaling. This novel observation invites the question of what serves as the endosomal receptors for double-stranded siRNAs. In acidic microenvironment such as the endosomes, double-stranded siRNA might dissociate and generate single-stranded siRNAs for intracellular signaling. In support of this hypothesis is the observation that certain double-stranded siRNAs are very less effective in inducing inflammatory cytokine production than their corresponding single-stranded sense or antisense strand counterparts (Figure 4). Depending on the relative internal stability, certain siRNA duplexes may exist in equilibrium with their single strand forms, which can activate TRL7 and/or TLR8 (Figure 5). Alternatively, double-stranded siRNAs may signal through endosomal TLR3, known to bind double-stranded RNAs.[21] Because TLR3 and 7 are not expressed by monocytes which responded to siRNAs, although less than adherent PBMC, the data would indicate that the induction of TNF-α by siRNA in monocytes is mediated by TLR8, which is preferentially expressed in these cells.

Given that single-stranded siRNAs are highly immunostimulatory compared to their corresponding double-stranded siRNAs, during siRNA annealing it is important to make sure that all siRNA molecules exist as siRNA duplexes. Although all double-stranded siRNA preparations used in this study were checked by PAGE for the presence of unannealed single-stranded siRNAs, some of the double-stranded siRNA effects could originate from tiny traces of free single-stranded siRNAs. Whatever the nature of double-stranded siRNA receptors, however, the present data indicate for the first time that induction of inflammatory cytokines and interferon-α by either double-stranded or single-stranded siRNAs requires endosomal acidification in fleshly isolated PBMC. Therefore, the development of carriers that deliver siRNA directly into the cytoplasm should overcome the problem of inflammatory cytokine induction by chemically synthesized siRNA, a major obstacle for systemic administration of siRNA in patients. Similarly to siRNA liposome complexes, naked siRNAs delivered *via* other techniques *in vitro* and *in vivo* are more likely to be taken up by cells *via* endocytosis. However, liposomal lipids may render siRNAs more immunostimulatory than the cellular receptors involved in nucleic acid uptake.

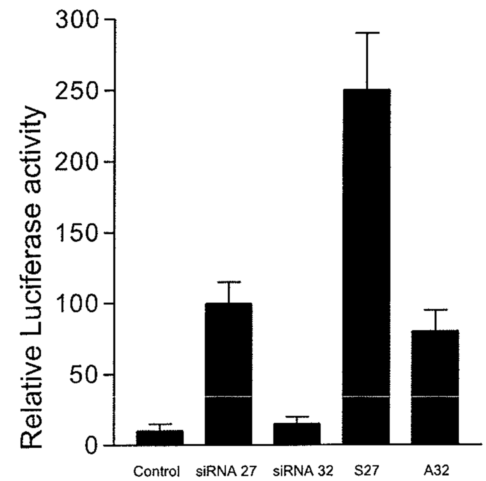

Figure 6. Immunostimulatory siRNAs activate the NF-κB transcription factor. A NF-κB-dependent luciferase reporter gene was co-transfected in human adherent PBMC with a control plasmid containing the *Renilla* luciferase gene under the control of the CMV promoter for 24 hours. Subsequently, cells were treated with either siRNA 27 or 32. The sense (S) and the antisense strand (A) of siRNA 27 and 32, respectively, were also tested. Eighteen hours later, luciferase activity was determined using Dual-Luciferase Reporter Assay System (Promega). The data show the relative firefly luciferase activity normalized to that of *Renilla* luciferase. Results are shown as means of three independent experiments ±SD.

The finding that several siRNA sense strands (mRNA sequences) can be highly immunostimulatory indicates that endosomal Toll-like receptors can recognize self-RNA. Thus, how can the immune system distinguish between self and non-self RNAs (e.g. viral RNAs,) and how to mount and amplify an immune response against invading RNA in a specific manner? The present data argue that the immune system uses the endosomes or lysosomal compartments as molecular recognition signature for RNA in general (whether self or non-self). In contrast to viral RNAs, self-RNAs exist in the cytoplasm and therefore cannot enter the endosomal compartments; hence in normal situation the immune system cannot be activated by self-RNAs. Similar to single-stranded siRNAs, liposomal delivery of short single-stranded RNA (21 nt) derived from HIV-1 TAT mRNA or 27 nt derived from human TNF-α mRNA also induced TNF-α and IFN-α production in adherent PBMC (data not shown). Thus, endosomal location of RNA seems to function as a "danger signal" for TLR recognition, and subsequent activation of innate immunity genes. The observation that certain sequences are more stimulatory than others would indicate that endosomal TLRs, in particular TLR8, have preferences for particular nucleotides and/or structures that need further investigation.

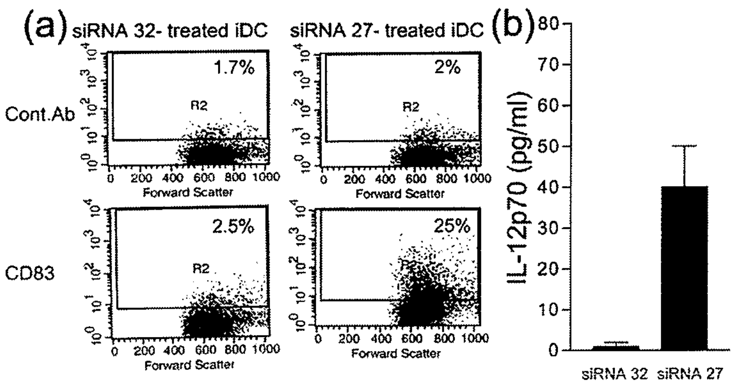

Figure 7. Immature DC activated with siRNA upregulated the expression of CD83 and secreted IL-12. Immature dendritic cells were cultured for 48 hours in the presence of either siRNA 27 or 32 (100 nM) and then stained with FITC-labeled monoclonal antibodies specific for CD83 (a). Cells were also stained with the corresponding isotype control. The mean fluorescence of stained cells is shown in the upper-right corner of the individual dot plots. Results are shown as means of three independent experiments ±SD. (b) After 48 hours stimulation with siRNAs, IL-12 was measured in the supernatants using ELISA. Results are shown as means of three independent experiments ±SD.

siRNA treatment can activate the NF-κB promoter

The core TLR signaling pathway uses myeloid differentiation factor 88 (MyD88) as the primary adaptor protein and results in NF-κB activation, cytokine production and expression of costimulatory molecules, such as the class II major histocompatibility complex. To address the involvement of NF-κB, promoter activation studies using firefly luciferase as a reporter gene were performed. Adherent PBMC were transiently transfected with NF-κB-luciferase gene along with plasmid DNA with the *Renilla* luciferase gene for 24 hours. Subsequently, the cells were either mock transfected or transfected with a stimulatory siRNA, and 18 hours later, cells were harvested, and equal amounts of cell extracts were subjected to luciferase assay (Figure 6). The data showed that double-stranded siRNA 27 could induce around eightfold increase in NF-κB activity, whereas 22-fold increase was obtained with the single-stranded siRNA (S27), again identifying single-stranded siRNAs as an important inducer of NF-κB activation.

Single-stranded and double-stranded siRNAs stimulate monocyte-derived immature dendritic cells to produce cytokines and up-regulate costimulatory molecules

If we view the induction of inflammatory cytokines as a beneficial mediator in cancer and infectious diseases, then immunostimulatory siRNAs could emerge as a viable agent to knock-down specific genes and activate innate and acquired immunity against tumor cells. Notably, stimulation of immature dendritic cells (iDC) by microbial products induces the production of inflammatory cytokines such as TNF-α and IL-12, which can induce differentiation of T cells into a T helper cell type 1.[26] In addition, these stimuli are known to up-regulate certain costimulatory molecules such as CD40, CD80, CD83 and CD86. This process underlies DC maturation, and it strongly potentiates the ability of DC to activate naive T cells.[26,27] Therefore, the effects of siRNAs upon the maturation of DC were investigated. To generate iDCs, freshly isolated monocytes were cultured for six days in the presence of GM-CSF (50 ng/ml) and IL-4 (100 ng/ml). Subsequently, they were both transfected with siRNA 27 or siRNA 32 and then analyzed by flow cytometry (Figure 7(a)). Adding immunostimulatory siRNA 27 to the culture medium upregulated the expression of CD83, whereas no significant effects were obtained with the control siRNA 32. Treatment with siRNA 27 also upregulated the expression of CD86 (data not shown). Furthermore, siRNA 27-treatment resulted in IL-12 production (Figure 7(b)). Similar results were obtained with single-stranded siRNA 27. These results suggest that immunostimulatory siRNAs can enhance immunity by inducing cytokine production and DC maturation. Thus, the use of immunostimulatory double-stranded or single-stranded siRNAs as a new generation of adjuvants may facilitate the design of effective vaccines.

The ability to enhance or augment the innate immune response clearly represents a potential powerful way to prevent or treat infections as well as a way to develop cancer vaccines. Recently, it has been discovered that unmethylated CpG dinucleotides in particular base contexts are recognized by the immune system as danger signals.[28] In animal models, deoxyoligonucleotides with stimulatory CpG motifs have been shown to be of therapeutic value as adjuvants for conventional and therapeutic vaccines against infectious diseases and tumors.[29] As shown in the present study, the immunostimulatory RNAs, in particular single-stranded siRNAs, activated human monocytes and dendritic cells to produce TNF-α and IL-12, two key cytokines in immune regulation. In addition, the induction of other cytokines such as IL-6 and interferons is an essential response for the clearance of viral infections.[30] Natural killer cells are activated by cytokines and chemokines, including type I IFN and IL-12, which are secreted by dendritic cells.[31] Incubation of whole PBMC with the

Table 2. Sequences of siRNAs targeting human TNF-α

SiRNAs	Sequence (5′–3′) (Sense strand)
H1	GAGUGACAAGCCUGUAGCCTT
H2	CUCAGCGCUGAGAUCAAUCTT
H3	CUAGUGGUGCCAGCCGAUGTT
H4	GCGUGGAGCUGAGAGAUAATT

immunostimulatory siRNAs also induced the expression of CD69 on T cells (data not shown). Therefore, treatment of cancer and infectious diseases might benefit from the induction of cytokine and dendritic cell activation by immunostimulatory siRNAs. TLR activators are already used as adjuvants to boost immune responses in vaccines. However, the success of a given vaccine may depend on appropriate activation of TLR. Therefore, single-stranded sense RNA-27 should be explored as an adjuvant for vaccination and immunotherapy. On the other hand, the development of TLR antagonists holds promise as a new class of anti-inflammatory agents. Although further study is required, some of the non-stimulatory RNAs may function as antagonists.

Selection of siRNA against human TNF-α

One of the key cytokines crucial to many biological processes is TNF-α. Low levels of TNF-α produced by monocytes and tissue macrophages under physiological conditions are expected to be involved in maintaining cellular and tissue homeostasis. The production of TNF-α increases in

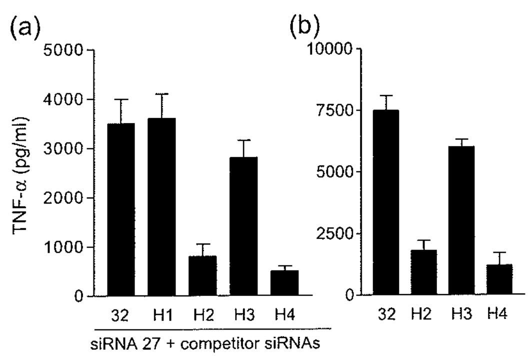

Figure 8. Selection of siRNAs against human TNF-α. (a) Inhibitory effects of TNF-α siRNAs on TNF-α production by the immunostimulatory siRNA 27. Adherent PBMC cells were cotransfected with siRNA 27 and various siRNA targeting the human TNF-α (H1-4). Eighteen hours later supernatants were analyzed for TNF-α contents by ELISA. Results are shown as means of three independent experiments ±SD. (b) Blockage of LPS-induced TNF-α expression by the inhibitory siRNAs. The cells were transfected for 18 hours with either siRNA 32, H2, H3 or H4 (50 nM). Subsequently, cells were stimulated with LPS (20 ng/ml) for eight hours and then supernatants were analyzed for TNF-α contents by ELISA. Results are shown as means of three independent experiments ±SD.

response to microbial infection and tissue injury. Because of its important role in the pathogenesis of a variety of inflammatory and immune diseases, TNF-α has been identified as a key target for pharmacological modulation.[32] Therefore, the development of agents that specifically inhibit TNF-α may provide clinicians with a valuable alternative to traditional disease-modifying anti-inflammatory drugs. In this respect, we have shown that siRNA-targeting mouse TNF-α can delay the onset of LPS-induced sepsis.[33] Out of 12 recently tested siRNAs targeting mouse TNF-α, siRNA 29 (see Table 1), exhibited the greatest protective effect.

To select siRNAs against human TNF-α, several siRNAs were designed (Table 2) and tested for their inhibitory effect on siRNA 27-induced TNF-α production in adherent PBMC (Figure 8(a)). Some siRNAs were effective in inhibiting TNF-α production, whereas others had no effects, thus suggesting the specificity of the inhibitory effects. When tested alone, the siRNA inhibitors significantly reduced LPS-induced TNF-α production by adherent human PBMC (Figure 8(b)). The utility of this selection assay may be extended to other inflammatory cytokines and type I interferon to select effective siRNAs in human blood cells. It is worth noting that the immunostimulatory effects of siRNAs are more pronounced in human PBMC as compared to other human cell lines. This may depend on the expression levels of Toll-like receptors, which is under investigation.

Collectively, the present study underscores for the first time the importance of the endosome compartments in siRNA intracellular signaling in adherent PBMC. The results can easily be explained by the interaction of RNA molecules with endosomal Toll-like receptors and subsequent induction of inflammatory and interferon responses (see Figure 5). The finding that endosomal localization of self RNA can trigger Toll-like receptors signaling in adherent PBMC is intriguing because it indicates that endosomal self RNA can display a molecular pattern capable for triggering innate immunity activation.

Experimental Procedures

siRNAs

The siRNAs used in this study correspond to various molecules targeting genes related to other ongoing projects. All siRNAs were chemically synthesized by Eurogentec (Seraing, Belgium), dissolved in water and annealed in the transfection buffer (20 mM Hepes, 150 mM NaCl, pH 7.4) at 20 μM. Analysis of LPS/endotoxin levels in siRNA stocks was found to be less than 0.01 EU/ml (PyrogentR, CAMBREX). The sequences of the sense strands and the target genes are shown in Table 1.

Preparation of human cells

Human mononuclear cells were prepared from buffy

coats by density gradient centrifugation (Lymphoprep, Nycomed Pharm, Oslo), washed, and then resuspended in RPMI 1640 containing 10% heat-inactivated fetal calf serum (FCS). Enriched monocyte populations were isolated by plastic adherence. After three hours incubation at 37 °C, non-adherent cells were removed by repeated gentle washing with warm medium. More than 75% of the obtained cells by this technique are CD14+ cells. In addition, CD14+ cell population was prepared from PBMC by positive selection with immunomagnetic beads coated with anti-CD14 monoclonal antibodies (Dynal, Oslo, Norway).

Culture of dendritic cells

Enriched populations of CD14 positive monocytes were isolated from human PBMC as described above and cultured for five days in RPMI 1640 supplemented with 10% FCS and antibiotics, in the presence of 50 ng/ml GM-CSF and 100 ng/ml IL-4 to obtain monocyte-derived immature dendritic cells (iDC). After five days in culture, the cells were either mock transfected or transfected with either siRNA 27 or 32 for 48 hours, and then cells were stained with FITC-conjugated monoclonal antibodies specific for CD83 (Immunotech). Cells were also stained with FITC-conjugated normal mouse IgG1, an isotype control.

FACS staining and analysis

Briefly, 2×10^5 cells were incubated in the recommended FITC-labeled antibody dilutions for 30 minutes at 4 °C in staining buffer (PBS containing 0.5% FCS and 0.1% azide). Cells were also stained with the corresponding Ab isotype controls. Subsequently, the cells were washed twice with the staining buffer and then analyzed on a FACScan using Cell-Quest software (BD Biosciences). Cells were gated in forward/side scatter and the quadrants were set on the relative isotype controls.

Transfection and ELISA

Cells were seeded at 5×10^4/well per 200 µl in 96-well plates. After an overnight incubation with siRNA/liposome complexes, culture supernatants were collected and cytokine contents were assessed by ELISA. Cells were transfected in RPMI 1640 medium (PAA Laboratories GmbH) supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 µg/ml penicillin and 100 µg/ml streptomycin using DOTAP (10 µg/ml) as described.[34] Each assay was carried out in triplicate. When naked siRNAs were used, cells were incubated in X-VIVO 15 medium without adding FCS (CAMBREX). In the case of monocytes, 2×10^4 cells were used per well. The inactivation of PKR was carried out as follows: Cells were incubated with 2-aminopurine (10 mM), a specific inhibitor of PKR, for one hour prior to addition of the siRNA/liposome complexes. After an overnight transfection with the tested molecules, the levels of TNF-α, IL-6 and IL-12p70 in the culture supernatants were measured by available ELISA kits according to the manufacturer's instructions (R&D Pharmingen). Samples were run in triplicate. Human IFN-α was measured using ELISA kit (PBL Biomedical Laboratories).

Transfection and reporter assays

Adherent PBMC (2×10^6 cells) were seeded onto a 24-well plate and transfected the following day with firefly luciferase gene (1 µg) under the control of the NF-κB-promoter and pRL-TK DNA (1 µg, Promega) encoding the *Renilla* luciferase gene. After 24 hours, the cells were transfected with siRNAs and then cells were harvested 18 hours later. Luciferase activities were measured using the Dual-Luciferase Reporter Assay System according to the manufacturer's instructions (Promega).

RT-PCR analysis

Total RNA was extracted from freshly isolated monocytes and monocytes-derived iDC using total RNA isolation kit (Pharmacia Biotech). DNAse I-treated RNA (10 µg) was reverse-transcribed with NotI-d(T)18 primer using the first-strand cDNA synthesis Kit (Pharmacia Biotech) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was performed on 3 µl of cDNA in 50 µl of final volume. The TLR3 primers are:

5′-ATTGGGTCTGGGAACATTTCTCTTC-3′ (forward primer)
5′-GTGAGATTTAAACATTCCTCTTCGC-3′ (reverse primer)

As a control, β actin mRNA was also amplified using the following primers:

5′-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3′ (forward primer)
5′-CGTCATACTCCTGCTTGGTGATCCACATCTGC-3′ (reverse primer)

After 35 cycles of amplification (one minute at 92 °C; one minute at 56 °C; one minute at 72 °C) samples were analyzed by electrophoresis on 1.5% (w/v) agarose gel and visualized by ethidium bromide staining.

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